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Patent No. 5,166,191 and in U.S. Serial No. 07/902,637 (PCT US92/06927). Certain formulations of human relaxin are described in allowed U.S. Serial No. 08/050,745.

5 The structure and biological function and
activity of the remaining members of the insulin-
related family have been extensively studied. See,
e.g. Robinson and Fritz, 1981, *Biol. Reprod.* 24:1032-
1041; Soder, et al., 1992, *Endocrinology* 131:2344-
10 2350; Luthman, et al., 1989, *Eur. J. Biochem*
180(2):259-65; Jhoti, et al., 1987, *FEBS Lett.*
219:419-425; Smit, et al., 1988, *Nature* 331:535-538.
Among the structural features shared between relaxin
and the remaining members of the insulin-related
15 family of hormones are molecular weight, a "two-chain"
structure comprising a B-chain, a connecting C-
peptide, and an A-chain, and the number and
disposition of disulfide links.

Despite these similarities, the proteins comprising the insulin-related family have been found to have distinct biological functions and activities. It has been reported that this distinction is in large part a consequence of differences between a few type-specific amino acid residues. For example, the difference between the glycine in position A14 of human type II relaxin and the isoleucine in the equivalent position (A10) of insulin is considered critical in distinguishing between the biological activity of the two proteins. Schwabe and Büllesbach, 1994, *FASEB J.* 8:1-2.

A protein having the structural characteristics of insulin, insulin-like growth factor (IGF) and relaxin has been isolated recently from Leydig cells of the testes. Burkhardt, et al., 1993, *Genomics* 20:13-19. This protein, designated as a Leydig cell-specific insulin-like peptide (Ley I-L), has been

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characterized as being "insulin-like" due to the genomic location of the gene encoding Ley I-L vis a vis the gene encoding insulin (as compared to the genomic location of the gene encoding either relaxin or IGF). Burkhardt, et al., 1993, *Genomics* 20:13-19.

The Ley I-L protein has been characterized also as insulin-like, rather than either IGF-like or relaxin-like, based upon the protein's C-peptide chain length. More specifically, the C-peptide length of the Ley I-L protein is 49 amino acids, as compared to the 35 amino acid length of proinsulin C-peptide, the twelve amino acid length of the known proIGF C-peptides and the over one-hundred amino acid C-peptide length of prorelaxin. Finally, Ley I-L has been designated insulin-like based on the observation that the protein is expressed exclusively in prenatal and postnatal testicular Leydig cells. Burkhardt, et al., *supra*.

On the basis of the protein's similarities to insulin and the source of such protein, it was reported that the Ley I-L protein is implicated in testicular function. *Id.*, Adham, et al., 1993, *J. Bio. Chem.* 268(35):26668-6672.

In consultation with the inventors of the present invention, Tashima, et al., 1995, *J. Clin. Endocrinal. Metab.* 80:707-710, have investigated the accuracy of previous reports providing that the Ley I-L gene was only expressed in Leydig cells. Specifically, Tashima, et al. investigated whether the Ley I-L gene was present and expressed in female reproductive tissues, the human corpus luteum, trophoblasts, fetal membranes and breast tissue. As with the case with H2 relaxin, Tashima, et al. determined that the Ley I-L protein can be found in human corpus luteum and trophoblast. Unlike H2 relaxin, however, Ley I-L was

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not found to be expressed in fetal membranes, decidua and breast tissue.

Neither the Burkhardt/Adham group nor the Tashima group have reported the biological function of the Ley I-L protein. Thus, while the structure of this putative Ley I-L protein has been identified, no correct activity or use was known for this protein until the present invention, which completed the discovery of RLF through the identification and proof of its utility.

3. SUMMARY OF THE INVENTION

The present invention is directed to synthesized or recombinant compositions derived from the deduced amino acid and nucleic acid sequences for human Ley I-L. In one embodiment of the present invention, the composition comprises the full-length amino acid sequence for relaxin-like factor (RLF). In another embodiment of the present invention, the composition comprises a RLF protein derivative wherein the protein is shortened at either or both its 3' and 5' ends of either or both the A- or B-chains. In one embodiment, the A chain may be as short as fifteen amino acids in length and the B chain may be as short as thirteen amino acids in length. In yet further embodiments of the present invention, the composition is radiolabelled or represents an analog of RLF having relaxin-like activity.

The present invention is further directed to the use of such compounds for the treatment of diseases and disorders which may be otherwise treated with relaxin, either alone, or in combination with relaxin or other relaxin-like agents, and formulations thereof. In one embodiment of the present invention, the diseases or disorders are related to the abnormal expression of collagen and/or fibronectin. More

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FIG. 3. FIG. 3 depicts the HPLC record of the purified RLF. Chromatography was performed on Synchropak RP-P (4.1 x 250 mm) using a linear gradient from 20 - 50% in 30 min (A:0.1% TFA in H₂O and B:0.1% TFA in 80% acetonitrile) at a flow rate of 1 ml/min.

FIG. 4. FIG. 4 depicts a comparison of the CD spectra of human relaxin, human RLF, and porcine relaxin.

FIG. 5. FIG. 5 depicts the elution record of the HPLC separation of an RLF tracer preparation. The largest peak is unmodified RLF and the shaded region is the major radioactive peak used as tracer. Chromatography was performed on Aquapore 300 (2.1 mm x 30 mm) using a linear gradient from 23% B to 34% B over 60 min (A: 0.1% TFA in H₂O and B: 0.1% TFA in 80% acetonitrile) at a flow rate of 0.1 ml/min.

FIG. 6. FIG. 6 depicts the tissue distribution of RLF receptors in female estrogen primed mice as measured in vitro in a receptor-binding assay.

FIG. 7. FIG. 7 depicts the bioactivity of an increasing amount of relaxin in the presence and absence of 5 µg of RLF per animal. The increase in symphyseal width was easily recognized.

FIG. 8. FIG. 8 depicts the bioactivity of an increasing amount of RLF in the presence of a uniform amount of human relaxin again shows relaxin enhancement.

FIG. 9. FIG. 9 depicts a comparative bioassay of relaxin, RLF, and an optimal dose of both. RLF alone does not cause symphyseal widening but the high dose of the mixture still improves upon the high dose of relaxin alone.

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Contrary to early reports, however, the biological function and activity of RLF is similar to relaxin and distinct from insulin. For example, notwithstanding the shift in amino acid sequence of the receptor interacting region between RLF and relaxin, RLF interacts with the mouse brain receptor to which relaxin binds.

A basis for the present invention is the inventors' unexpected discovery that the previously isolated but uncharacterized RLF protein binds specifically to crude membrane preparations of mouse uterus and brain and shows crossreactivity with the relaxin receptor, but not the insulin receptor.

The deduced amino acid sequence for RLF would have predicted an opposite result because the critical Arg XXX Arg sequence in RLF is offset toward the C-terminal end of the B chain by exactly one turn of the helix. Thus, although RLF projects the arginines at nearly right angles away from the molecular surface in the manner of relaxin, one would expect that shifting the whole receptor-binding site would present quite a different binding environment to the receptor.

Notably, although RLF binds to the relaxin receptor, it does not appear to competitively bind to the relaxin receptor, *vis a vis* relaxin, except at higher concentrations. Rather, RLF appears to stimulate relaxin response. Thus, RLF can play an important supportive role for the relaxin action in humans. In addition, preliminary experiments suggest that RLF plays a role independent of relaxin in the male gonads.

In addition, although relaxin-like activity has historically been considered in terms of softening the pubic and cervical ligaments in preparation for parturition, it has also been shown to directly effect cells outside of the reproductive system. For

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example, consistent with relaxin, RLF may also be instrumental in inhibiting collagen and/or fibronectin overexpression and diseases related thereto (e.g. scleroderma)

5 Moreover, although RLF possesses relaxin-
enhancing properties, as described herein, RLF possess
independent and potentially additional biological
activity.

5.3. RLF Derivatives And Analogs

10 Following the present identification of RLF
as a protein having relaxin-like (rather than insulin-
like) activity, to the extent that RLF shares primary
and secondary homology to relaxin, as well as insulin,
identification of biologically active derivatives and
15 analogs of relaxin evidences the identity of
biologically active RLF derivatives and analogs.
Active relaxin analogs and derivatives have been
identified to include, for example, shortening either
or both the 5' and 3' end of the protein. See e.g.,
20 U.S. Patent No. 5,023,321. The present invention is
therefore directed to biologically active RLF
derivatives wherein the 5' and/or 3' end of the
protein has been shortened. See, above referenced
patents.

25 Importantly, it has been observed in human
relaxin that the arginines at positions B13 and B17
and potentially the amino acids of the first helix
turn in the midregion of the B-chain (Arg-Glu-Leu-Val-
Arg) are necessary or important to relaxin activity.
30 Other RLF analogs and derivatives may be obtained
using known techniques and this structural information
regarding relaxin.

Whether the RLF derivative or analog possesses relaxin-like activity and/or utility may be determined using assays known in the art for detecting relaxin activity. For example, bioassays used for the

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measurement of active relaxin during pregnancy and non-pregnancy, as described in Steinetz et al., 1960, *Endocrinology* 67:102-115 and Sarosi et al., 1983, *American Journal of Obstetrics and Gynecology* 145:402-405, may be used.

Similarly, specific immunoassays to detect for the presence of proteins having relaxin-like activity may also be used. See e.g., Sherwood et al., 1975, *Endocrinology* 107:691-696; O'Bryne and Steinetz, 1976, *Proceedings of the Society for Experimental Biology and Medicine* 152:272-276. The presence and activity of synthetic analogs of human relaxin comprising one or more accessible tyrosines (permitting direct iodination) may also be tested using a radioimmunoassay (RIA). Eddie et al., 1986, *Lancet* 1:1344-1346.

Each of the above-described assays, however, are limited in their application. Thus, as set forth below and as described in more detail in a co-pending application, filed concurrently herewith and entitled "Relaxin Diagnostic Assays And Kits," additional assays may also be used to assay RLF to determine the protein's activity and preferred applications.

5.4. Production Of RLF

RLF may be produced using techniques previously disclosed as useful in producing relaxin and insulin. For example, the cDNA for RLF disclosed in Burkhardt, et al., 1994, *Genomics* 20:13-19 and Adham, et al., 1994, *J. Biol. Chem.* 268:26668-26672 may be used to recombinantly produce RLF according to processes previously described as useful in recombinantly manufacturing relaxin (e.g., U.S. Patents Nos. 4,758,516, 4,871,670, 4,835,251 and co-pending U.S. Serial Nos. 07/908,766 (PCT US90/02085) and 08/080,354 (PCT US94/0699)). Similarly, such sequence information may be used to synthesize RLF

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amino acid substitutions for methionine that did not affect biological activity. Johnston, et al., in Peptides: Structure and Function, Proc. Ninth American Peptide Symposium, Deber, C.M., et al. (eds.)
5 (Pierce Chem. Co. 1985).

5.5. Indications/Methods Of Use

In vitro, proteins having relaxin-like activity decrease collagen synthesis by human dermal and synovial fibroblasts upregulated to overexpress
10 collagen with transforming growth factor-beta (TGF-beta) or interleukin-1, and by fibroblasts constitutively overexpressing collagen obtained from scleroderma patients. For example, relaxin decreases collagen accumulation in vivo in two rodent models of
15 fibrosis. Relaxin or relaxin-like proteins also increase the secretion of the collagenolytic metalloproteinase, collagenase, and also down-regulates the expression of the metalloproteinase inhibitor, tissue inhibitor of metalloproteinases.
20 Relaxin has been implicated consequently in the treatment and diagnosis of various diseases and disorders. For example, studies provide evidence that relaxin is effective in the treatment of scleroderma, sinus bradycardia, cardiovascular disease,
25 neurodegenerative and neurologic disorders, hair loss, depression. See e.g., U.S. Patent No. 5,166,191; U.S. Serial No. 07/902,637 (PCT US92/069); U.S. Applications entitled "Method For Treatment Of Hair Loss" and "Method For Treatment Of Depression," both
30 of which are filed concurrently herewith. Evidence also suggests the use of relaxin in diseases and disorders related to the abnormal expression of collagen or fibronectin, such as scleroderma or rheumatoid arthritis.

35 As provided herein, RLF possesses relaxin-like biological activity and is therefore similarly

A therapeutically effective dose refers to that amount of the compound that results in amelioration of symptoms or a prolongation of survival in a patient. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between LD50 and ED50. Compounds which exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g. Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 pl).

35 Administration of RLF, with retain or other
active agents, can be via any of the accepted modes of

While human dosage levels for treating many of the above-identified relaxin-related diseases or disorders have yet to be optimized for RLF, administered alone or in combination with relaxin, generally, a daily dose is from about 0.1 to 500.0 $\mu\text{g/kg}$ of body weight per day, preferably about 6.0 to 200.0 $\mu\text{g/kg}$, and most preferably about 12.0 to 100.0 $\mu\text{g/kg}$, depending on whether RLF is administered alone or in combination with relaxin. Generally it is sought to obtain a serum concentration of RLF, alone or in combination with relaxin, approximating or greater than normal circulating levels in pregnancy, i.e., 1.0 ng/ml, such as 1.0 to 20 ng/ml, preferably 1.0 to 20 ng/ml.

For administration to a 70 kg person, the dosage range would be about 7.0 μ g to 3.5 mg per day, preferably about 42.0 μ g to 2.1 mg per day, and most preferably about 84.0 to 700.0 μ g per day. The amount of RLF administered will, of course, be dependent on the subject and the severity of the affliction, the manner and schedule of administration and the judgment of the prescribing physician. One treatment regimen can employ a higher initial dosage level (e.g., 100 to 200 μ g/kg/day) followed by decreasing dosages to achieve steady relaxin or relaxin-like serum concentration of about 1.0 ng/ml. Another treatment regimen, particularly postpartum depression, entails administration of an amount of relaxin sufficient to attain normal pregnancy levels of relaxin (about 1.0 ng/ml) followed by gradual decreasing dosages until relaxin serum levels are no longer detectable (e.g. less than about 20 picograms/ml), optionally discontinuing treatment upon reaching that dosage level.

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In employing RLF, either alone or in combination with relaxin, for treatment of the above conditions, any pharmaceutically acceptable mode of administration can be used. RLF can be administered either alone or in combination with other pharmaceutically acceptable excipients, including solid, semi-solid, liquid or aerosol dosage forms, such as, for example, tablets, capsules, powders, liquids, gels, suspensions, suppositories, aerosols or the like. Relaxin can also be administered in sustained or controlled release dosage forms (e.g., employing a slow release bioerodable delivery system), including depot injections, osmotic pumps (such as the Alzet implant made by Alza), pills, transdermal (including electrotransport) patches, and the like, for prolonged administration at a predetermined rate, preferably in unit dosage forms suitable for single administration of precise dosages. The compositions will typically include a conventional pharmaceutical carrier or excipient and RLF. In addition, these compositions may include other active agents, carriers, adjuvants, etc.

In a preferred aspect of the invention, a sustained/controlled release RLF formulation was a selectively permeable outer barrier with a drug dispensing opening, and an inner RLF-containing portion designed to deliver dosage of RLF progressively diminished as a predetermined rate (e.g. containing about 30 mg of RLF in a matrix for delivery of initially about 500 μ g per day diminishing as a rate of 10 μ g per day.

In another preferred aspect of the invention, a sustained/controlled release RLF formulation has a selectively permeable outer barrier with a drug dispensing opening, a first inner relaxin-containing portion designed for steady state release of relaxin

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at a therapeutically effective daily dosage (e.g. containing about 50 mg of relaxin in a matrix for continuous delivery of about 500 μ g per day), and a second inner RLF-containing portion designed to
5 deliver a dosage of RLF progressively diminishing at a predetermined rate (e.g. containing about 3 mg of relaxin in a matrix for delivery of initially about 500 μ g per day diminishing at a rate of 50 μ g per day) commencing upon exhaustion of the relaxin from the
10 first inner portion.

Generally, depending on the intended mode of administration, the pharmaceutically acceptable composition will contain about 0.1% to 90%, preferably about 0.5% to 50%, by weight of RLF, either alone or
15 in combination with relaxin, the remainder being suitable pharmaceutical excipients, carriers, etc. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see *Remington's Pharmaceutical*
20 *Sciences*, Mack Publishing Company, Easton, Pennsylvania, 15th Edition, 1975. The formulations of human relaxin described in U.S. Serial No. 08/050,745 are particularly preferred.

In cases of local administration or selective
25 uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction,
30 the manner of administration and the judgment of the prescribing physician.

5.6.2. Routes Of Administration.

Suitable routes of administration may, for example, include oral, rectal, transmucosal,
35 or intestinal administration. Parenteral administration is generally characterized by

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injection, either subcutaneously, intradermally, intramuscularly or intravenously, preferably subcutaneously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or emulsifying agents, pH buffering agents, solubility enhancers, and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, cyclodextrins, and the like.

The percentage of RLF and/or relaxin contained in such parenteral compositions is highly dependent on the specific nature thereof, as well as the needs of the subject. However, percentages of active ingredient of 0.01% to 10% in solution are employable, and will be higher if the composition is a solid which will be subsequently diluted to the above percentages. Preferably the composition will comprise 0.2-2% of the RLF, alone or in combination with relaxin in solution.

A more recently devised approach for parenteral administration employs the implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained. See, e.g., U.S. Patent No. 3,710,795.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tissue-specific antibody. The

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liposomes will be targeted to and taken up selectively by the tissue.

5.6.3. Composition/Formulation.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

10 Pharmaceutical compositions for use in accordance
with the present invention thus may be formulated in
conventional manner using one or more physiologically
acceptable carriers comprising excipients and
auxiliaries which facilitate processing of the active
15 compounds into preparations which can be used
pharmaceutically. Proper formulation is dependent
upon the route of administration chosen.

The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such

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as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the
5 preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal
10 compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a
15 depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic
20 materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for the hydrophobic compounds of the invention is a cosolvent system
25 comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system. VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v
30 polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic compounds well, and itself produces low
35 toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied

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considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical compounds may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the compounds may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various of sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent, additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

35 Formulations of RLF may also be administered to
the respiratory tract as a nasal or pulmonary

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inhalation aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose, or with other pharmaceutically acceptable excipients. In such a case, the particles of the formulation may advantageously have diameters of less than 50 microns, preferably less than 10 microns. See, e.g., U.S. Patent No. 5,364,838, which discloses a method of administration for insulin that can be adapted for the administration of RLF, alone or in combination with relaxin in the present invention.

RLF for treatment of such disorders such as alopecia, may also be administered topically in a formulation adapted for application to the scalp, such as a shampoo (e.g., as disclosed in U.S. Patent No. 4,938,953, adapted according to methods known by those skilled in the art, as necessary for the inclusion of protein ingredients) or a gel (e.g., as disclosed in allowed U.S. Serial No. 08/050,745) optionally with increased relaxin concentrations to facilitate absorption.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize

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starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g.,

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dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

10 5.6.4. Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labelled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of depression, sinus bradycardia, hair loss, neurologic or neurodegenerative diseases, scleroderma, cardiovascular disease or disorders or diseases related to uncontrolled or abnormal collagen or fibronectin formation.

More specific dosage, formulation and methods of administration may be derived from information contained in U.S. Patent Nos. 5,166,191, U.S. Application Serial Nos. 07/902,637 (PCT US92/06927) and 08/050,745 (allowed), and co-pending applications, filed concurrently herewith, entitled "Method Of Treatment For Hair Loss" and "Method Of Treatment For Depression."

The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention.

5 They should not be considered as limiting the scope of the invention, but merely as being illustrative and representative thereof.

10 As described above, RLF may be produced by isolating the protein from natural sources, synthesizing the protein based on RLF's deduced amino acid sequence and recombinantly manufacturing the protein based upon available cDNA data.

15 One procedure for synthesizing RLF is as follows:

Materials. L-Amino acid derivatives for peptide synthesis were purchased either from Bachem Bioscience (Philadelphia, PA) or Bachem California (Torrance, CA). Solvents for peptide synthesis and chromatography were distilled in glass (Burdick and Jackson; Muscagon, MI), and the chemicals for peptide synthesis were obtained from Perkin Elmer Applied Biosystems (Foster City, CA). Other chemicals of analytical grade were used without further purification.

Methods. The following method was followed to synthesize RLF:

Peptide Synthesis: The B chain of the RLF protein was synthesized by tert-butyloxycarbonyl¹-chemistry using conventional HF-labile side chain-protecting groups for all three functional amino acids except cysteines. Cysteine B10 was protected by the acetamidomethyl group and B23 by the thiol-protecting/activating group [S-(3-nitro-2-pyridinesulfonyl)] (CysB23). Methionine was protected by sulfoxide formation, and tryptophan by the

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N(in)formyl group. The synthesis was performed on an Applied Biosystems peptide synthesizer model 430A on [4-(oxymethylphenylacetamidomethyl)resin loaded with 0.4 mmol tert-butylloxycarbonyl-alanine. Deprotection and removal from the solid support was accomplished by HF-treatment in the presence of 5% m-cresol. The crude peptide was extracted with 20% acetic acid and lyophilized (yield 1.387 g). The B chain was purified on Sephadex G50-sf (2.5 cm x 50 cm) in 1 M acetic acid (yield: 840 mg), followed by preparative HPLC on Synchropak RP-P (2.1 cm x 25 cm) in portions of 50 to 70 mg. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in 80% acetonitrile (solvent B). The column was equilibrated in 20% B and the peptide eluted with a linear gradient of 20% B to 50% B over 1 h at a flow rate of 5 ml/min (overall yield: 233 mg). Amino acid composition: Thr 2.00 (2); Ser 0.86 (1); Glu 2.90 (3); Gly 3.28 (3); Ala 2.16 (2); Cys 0.89 (2); Val 3.19 (3); Met 1.22 (1); Leu 1.94 (2); Phe 0.99 (1); His 2.44 (2); Lys 0.96 (1); Arg 3.81 (4).

The A chain (0.25 mmol) was synthesized via Fast-moc chemistry on an ABI peptide synthesizer (model 430A) on p-benzyloxybenzyl resin. All side chains were protected by TFA-labile protecting groups except Cys(A11), which was acetamidomethyl-protected, and Cys(A24) which was protected by the HF-labile p-menthylbenzyl group. The A chain was deprotected with TFA/thiophenol (10:1 v/v), using 50 mg peptidyl resin/ml for 90 min at room temperature (5). The TFA was evaporated and the peptide precipitated with ether. The precipitate was collected by centrifugation, the supernatant discarded, and the pellet washed twice with ether and air-dried. The peptide was suspended in water, dissolved by the addition of ammonia, and desalted on Sephadex G25-m in

5. After completion of the disulfide bond formation the A chain was dialyzed against water and lyophilized (yield 372.3 mg). Aliquots of 20 mg were further purified by preparative HPLC on Synchropak RP-P (10 mm x 250 mm), using 0.1% TFA in water for solvent A and 0.1% TFA in 80% acetonitrile for solvent B. The column was equilibrated in 30% B and the peptide eluted with a linear gradient of 30% B to 50% B over 30 min at a flow rate of 3 ml/min (overall yield: 166.5 mg). Amino acid composition: Asp 2.20 (2); Thr 3.00 (3); Ser 0.99 (1); Glu 1.92 (2); Pro 2.25 (2); Gly 1.06 (1); Ala 4.18 (4); Cys 1.62 (4); Leu 3.60 (4); Tyr 1.82 (2); Arg 0.98 (1).

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min at a flow rate of 3 ml.min (yield: 34.1 mg, 54.8%).

The resulting peptide contained acetamidomethyl groups in positions Cys A11 and Cys B10, the N(in) formyl group in Trp B27, and a sulfoxide in the side chain of Met B5. For the formation of the third disulfide bond the peptide (9.3 mg) was dissolved in water (3.5 ml) and added to a stirred solution consisting of acetic acid (3.5 ml) 6 N HCl (19.1 μ l) and 3 ml of 50 mM iodine in acetic acid (8). The reaction was performed at room temperature for 10 min, quenched with ascorbic acid, and the product was desalted on Sephadex G25-sf in 1 M acetic acid and lyophilized. After purification by preparative HPLC (conditions as before) (yield: 3.42 mg, 36.8%) the protein still contained protecting groups in Trp (B27) and Met (B5).

Complete deprotection was achieved first by treatment of 11.3 mg of the peptide with 2 ml of water/piperidine 9:1 (v/v) for 2 min at room temperature. The base was neutralized with 0.4 ml acetic acid and the peptide purified by preparative HPLC, dried (yield:11.0 mg, 97.5%), and 10 mg of peptide-containing methionine sulfoxide was reduced with 1 ml of TFA/0.5 M NH_4I in water 9:1 v/v for 15 min at 0°C. Free iodine was reduced with 0.5 M ascorbic acid in water and the reaction quenched by dilution with water. The final peptide was recovered by preparative HPLC (conditions as before) (yield 7.57 mg=75.7%). Amino acid compositions: Asp 2.02 (2), Thr 4.79 (5), Ser 1.77 (2), Glu 4.86 (5), Pro 5.17 (5), Gly 4.15 (4), Ala 6.09 (6), Cys 3.51 (6), Val 2.86 (3), Met 0.70 (1), Ile 0 (0), Leu 5.74 (6), Tyr 2.12 (2), Phe 0.98 (1), His 2.00 (2), Lys 1.26 (1), Trp 1.00 (1), Arg 5.02 (5). (overall yield 14.9%).

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The mobile phase of all HPLC systems used consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in 80% acetonitrile (solvent B).

5 For preparative HPLC, a Waters HPLC system consisting of two pumps (model 6000A) and gradient programmer (model 680) was used in combination with a Synchropak RP-P column (C18) (SynChrom, In) and an Uvicord S UV (226 nm) monitor (LKB, Bromma Sweden).

10 Usually 1 to 20 mg of peptide was separated using linear gradients as indicated.

Analytical HPLC I was performed on Aquapore 300 (C₈; 2.1 mm x 30 mm) using an Applied Biosystems HPLC model 130A. Separation was achieved with a linear gradient from 23% to 34% B in 60 min at a flow rate of 0.1 ml/min. The peptide was detected by UV absorbance at 230 nm.

Analytical HPLC II was performed on Synchropak RP-P (C18, 4.1 mm x 250 mm) using a Waters HPLC system. Separation was achieved with a linear gradient from 20% B to 50% in 30 min at a slow rate of 1 ml/min. The peptide was detected by UV absorbance at 220 nm. The above-described HPLC may also be used to verify the purity of the RLF, as set forth in FIG. 3.

The primary structure of RLF, as synthesized according to the above procedure, or any other solid phase methodologies in combination with site-directed sequential disulfide bond formation (a schematic depicting said formation is set forth at FIG. 2), is set forth at FIG. 1.

Protein Confirmation And Verification. The identity of the synthesized RLF may be confirmed and verified according to known techniques.

35 Amino Acid Analyses: Following protein
synthesis and purification, amino acid analyses was

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conducted to confirm the protein's identity. First, peptides were hydrolyzed in vapor phase 6N HCl containing 0.1% phenol for 1 h at 150°C. The amino acids were detected after pre-column modification with phenylisothiocyanate and separation by HPLC (Pico-Tag system, Waters Millipore).

Sequence Analyses: The identity of the protein was verified by sequence analysis. Specifically, such analyses were performed on an ABI 477 A pulsed liquid protein sequencer and an in-line ABI 120A phenylthiohydantoin analyzer (ABI, Applied Biosystems, Foster City, CA). Chains were prepared by reduction of about 10 μ l of the relaxin-like factor in 20 μ l 50 mM DTT in 3 M guanidinium chloride, 0.2 M Tris, HCl at pH 8.5 for 1 h at 37°C, diluted with 30 μ l of solvent A, followed by separation on Aquapore 300 (see: Analytical HPLC for conditions).

Upon reduction two chains were generated, isolated, and the subsequent sequence analyses of both chains showed the desired structure.

UV Spectroscopy: The confirmation and structure of the synthesized protein was then confirmed by UV spectroscopy. Such spectroscopy was performed on an OLIS Cary 15 spectrophotometer conversion (On-Line Instrument Systems Inc., Bogart, GA). UV-spectroscopy was used to determine the protein concentrations of the RLF protein. The specific absorption coefficient ($\epsilon_{276}=1.40 \text{ cm}^2/\text{mg}$) was obtained by direct comparison of UV absorbance and the recovery of amino acids after hydrolysis and amino acid analysis.

No partial hydrolysis at the acid labile Asn-Pro bond was detected.

Circular Dichroism: Further confirmation of the synthesized protein's identity was performed by CD spectroscopy, as performed on a Jasco J-710

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spectropolarimeter using a cell of 0.02 cm path length. Proteins were dissolved in 25 mM Tris/HCl at pH 7.7 and concentrations were determined by UV spectroscopy: 0.67 mg/ml for porcine relaxin, 0.54 mg/ml for relaxin-like factor, and 0.55 mg/ml for human relaxin. Spectra were measured at a resolution of 0.2 nm, a band width of 2 nm, and 5 spectra were averaged. Molar ellipticity was calculated according to Adler et al. (9) using mean residual weights of 110.4 for relaxin-like factor, 113.6 for porcine relaxin, and 112.5 for human relaxin.

Comparative measurements of circular dichroic spectra suggested near identity of the solution structures of RLF and porcine relaxin. See, FIG. 4.

15 Mass Spectrometry: Finally, mass spectra
were recorded on a JEOL HX110/HX110 4 sector tandem
mass spectrometer (JEOL, Tokyo, Japan) to verify the
protein's identity and proper synthesis. Samples were
dissolved in 0.1% trifluoroacetic acid at a
20 concentration of about 0.8 nmol/ μ l.

Mass spectrometry showed the correct mass ion for the synthetic RLF (found: 6294.6, theoretical 6293.2).

6.2. Production Of Labelled RLF

¹²⁵I-labeled RLF, containing side chain-protected tryptophan and methionine, may be prepared according to the above procedure wherein the synthesized peptide (10 µg in 5 µl of water) is then placed into a 200 µl Eppendorf vial and 5 µl phosphate buffer (250 mM, pH 7.4), followed by 2 µl of ¹²⁵I-(1 mCi), and 5 µl of chloramine T (2 mg/ml in phosphate buffer pH 7.4) are added. The reaction was performed for 1 min on ice, quenched by addition of 5 µl of sodium thiosulfate (5 H₂O) (50 mg/ml in phosphate buffer pH 7.4), and 5 µl of NaI (20 mg/ml in phosphate buffer pH 7.4). The side chain-protecting group of Trp was removed by addition of 5 µl of piperidine.

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After 2 min at room temperature the reaction was quenched by the addition of 5 μ l of glacial acid, the reaction mixture was diluted with 10 μ l of water and loaded onto a Aquapore 300 column for separation. The protein was detected by UV absorbance and peaks were manually collected into 100 μ l of 1% bovine serum albumin in water.

The labelled RLF may be used as an RLF tracer which could then be used to separate by HPLC the different RLF derivatives to yield a carrier-free tracer. See, FIG. 5. Alternatively, such labelled RLF may also be used in binding assays and for RLF receptor mapping.

6.3. Receptor Binding Assay

Insulin-receptor binding assays were performed on crude membrane preparations of term placenta, as described in Hock and Hollenberg, 1980, *J. Biol. Chem.* **255**:10731-10736, using 125 I-iodo-Tyr^{Ala} porcine insulin as tracer according to the method of Linde, et al., 1986, *J. Chromatogr.* **369**:327-339.

The assays were performed in HMS-buffer (25 mM HEPES, 104 mM NaCl, 5 mM MgCl₂, 0.2% bovine serum albumin; pH 7.4) in a total volume of 100 μ l. Labeled insulin (50,000 cpm/assay, 150 pM) and variable amounts of insulin were incubated with crude membranes for 1 h at room temperature. Thereafter 1 ml of buffer was added, the membranes collected by centrifugation in a microcentrifuge at 14,000 rpm for 5 min, the supernatant discarded, and the tip of the Eppendorf vial cut off and counted in a γ -counter (Minigamma, LKB, Sweden). To determine nonspecific binding unlabeled insulin was used at a concentration of 2 μ l/ml (0.33 μ M) and nonspecific binding was usually below 10% of the total binding.

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μM). In a typical experiment the specific binding was between 25% and 40% of the total binding.

Tissue specificity was determined using crude membrane preparations of leg muscles, kidneys, liver, 5 brain, and uterus (of estrogen primed mice). The crude membranes were prepared as described for relaxin. Binding was based on protein concentration determined by Lowry.

According to the above-described assay, a 10 hundred-fold excess of human RLF displaces 50% of the relaxin tracer from a mouse brain relaxin receptor preparation. The difference in affinity is still within the range of specific binding, i.e., several orders of magnitude better than the binding of insulin 15 or guinea pig relaxin to this receptor (see generally, Büllesbach, et al., 1994, *Endocrine*, 2:1115-1120) indicating that RLF recognizes the relaxin receptor.

As discussed above, this result was surprising because the critical Arg XXX Arg sequence in RLF is 20 offset toward the C-terminal end of the B chain by exactly one turn of the helix (See, FIG. 1).

Of the tissues tested with ^{125}I -RLF as tracer such as brain, uterus, skeletal muscle, kidney and liver, only the brain- and the uterus membrane preparation 25 showed specific binding (See, FIG. 6). These are tissues that also bind relaxin in a competitive and saturable manner. To test for crossreactivity the assays were performed with tracers and competitive

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cold molecules exchanged. The results from such assay are set forth below at Table 1:

TABLE 1
Relaxin-Binding Assay Results

Tissue (mouse)	Tracer	Competitor	50% Binding	Range
Brain	pRLX	hRLX	1 ng	0.1-2.0
Brain	pRLX	hRLF	200 ng	190-220
Uterus	pRLX	hRLX	1 ng	0.1-2
Uterus	pRLX	hRLF	10000 ng	--
Uterus	hRLF	hRLX	800 ng	600-1000
Uterus	hRLF	hRLF	0.3 ng	0.1-0.6
Brain	hRLF	hRLX	1000 ng	900-1100
Brain	hRLF	hRLF	0.3 ng	0.1-0.6

pRLX = porcine relaxin, hRLX = human relaxin,
pRLF = porcine RLF and hRLF = human RLF

These results suggest strongly that RLF does have its own receptor in these tissues and that the relaxin receptor is recognized by RLF, but with a significantly lower affinity than relaxin.

Furthermore, the data support that the brain and uterine relaxin receptors differ with respect to crossreactivity. Specifically, the uterine relaxin receptor barely recognizes RLF whereas the brain receptor shows moderate crossreactivity. In general, the RLF receptor binds its substrate with greater affinity than the relaxin receptor displays toward relaxin.

6.5. Sperm Motility Assay

Relaxin and proteins having relaxin-like activity may be identified by a sperm motility assay.

Materials And Methods. Semen is obtained by masturbation from healthy volunteers. The sample is allowed to liquefy at room temperature and is then mixed with Minimum Essential Medium (MEM) with Hepes buffer added. This medium is used because it coincides with that washing medium employed by the in

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All the above compounds were compared against stingray alkaline gland fluid (ALG) from the HPLC. AGF significantly increased then maintained sperm motility for the first two time periods then was
5 approximately 10% higher for the last three time periods.

6.6. In Vitro Inhibition Of Collagen and
Fibronectin Expression By Human Lung
Fibroblasts

Whether RLF inhibits collagen and
10 fibronectin expression has been studied in the context of human lung fibroblasts. Specifically, RLF (1-100ng/ml) was applied to human lung fibroblasts in serum-free medium and assayed for collagen secretion by biosynthetic labelling with 3 H-proline in the
15 presence of ascorbate and B-aminopropionitrile. When tested on lung fibroblasts stimulated with TGF- β , RLF's ability to inhibit collagen expression at various dose levels can be determined. The presence of another extracellular matrix molecule, fibronectin,
20 in conditioned media was assessed by Western immunoblotting using an anti-fibronectin polyclonal antibody, as well as biosynthetic labeling.

6.7. In Vitro Inhibition Of Collagen and
Fibronectin Expression By Synovial
Fibroblasts

25 Trauma to the shoulder or surgical intervention of large joints is often associated with limitation in mobility, in many cases due to an exaggerated fibrotic response to synovial or capsular tissue. Extracellular matrix-producing cells, such as
30 synovial fibroblasts, are capable of the extremes of degradation or repair. The overproduction of collagens, fibronectin and other extracellular matrix molecules can be due to the local expression of
35 cytokines, such as transforming growth factor TGF- β . To the extent it has been demonstrated that relaxin

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can decrease TGF- β -stimulated collagen expression in a dose-dependent manner, up to 30% at a relaxin dose of 100ng/ml and fibronectin expression by 30%, RLF is also implicated in the modulation and control of collagen and fibronectin expression.

To test such hypothesis (to determine RLF's ability to down-regulate collagen and fibronectin expressed by synovial fibroblasts), fibroblasts can be explanted from pieces of rheumatoid synovium and treated with TGF-beta (1ng/ml) to stimulate expression of types I and III collagens. TGF-beta upregulated collagen expression at the protein level, as measured by biosynthetic labelling with ^3H -proline incorporation. More specifically, the following experiments were conducted to determine RLF's ability to modulate the expression of collagen, fibronectin and procollagenase in human synovial fibroblasts:

6.7.1. Assay To Determine The Inhibition Of Collagen Expression

The method for detecting and measuring collagen formation in the presence of relaxin described in Unemori and Amento, 1990, *J. Biol. Chem.* 265:10681-685 has been modified as follows to determine the ability of RLF, *in vitro*, to modulate the expression of collagen.

Materials And Methods. Rheumatoid synovial fibroblasts (Strain No. RSF64) were seeded at a density of 6.25×10^4 cells/cm² in tissue culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. After 24 hours, the cells were washed and treated with DMEM supplemented with 0.2% lactalbumin hydroxylsate with relaxin, RLF and/or transforming growth factor (TGF- β).

The cells were simultaneously biosynthetically labelled with ^3H -proline (25 $\mu\text{Ci/ml}$) in the presence of

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commercially available polyclonal anti-fibronectin antibody (Promega). The fibronectin band was scanned densitometrically to estimate levels of expression.

Experimental Results. Using the above protocol, it was determined that RLF decreases fibronectin expression independently. Specifically, addition of RLF (100 ng/ml) to the TGF- β -treated fibroblasts decreased TGF- β -stimulated fibronectin expression by 17%.

10 **6.8. In Vitro Stimulation Of Procollagenase Expression By Synovial Fibroblasts**

A method for detecting and measuring procollagenase formation is described in Unemori, et al., 1991, *J. Biol. Chem.* 266:23477-482. Such method was modified to measure the expression of procollagenase in the presence of RLF as follows:

15 **Materials And Methods.** Rheumatoid synovial fibroblasts (Strain No. RSF112) were seeded at a density of 6.25×10^4 cells/cm² in tissue culture dishes in DMEM supplemented with 10% fetal bovine serum. After twenty-four hours, the cells were washed and treated with DMEM supplemented with 0.2% lactalbumin hydroxylate with relaxin at 1, 10 and 100 ng/ml for 48 to 72 hours. Conditioned media were collected and an aliquot analyzed by gelatin zymography. Procollagenase was identified as a gelatinolytic doublet at 52/57 kDa. The intensity of the doublet (i.e., the amount of procollagenase expressed, was quantified by scanning densitometry.

25 **Experimental Results.** RLF stimulated expression of procollagenase in a dose-dependent manner comparable to that induced by relaxin. RLF at 1, 10 and 100 ng/ml stimulated procollagenase expression by 0, 2.0 and 4.2-fold. Relaxin induced procollagenase expression by 0, 1.6 and 4.9-fold at the equivalent doses.

PATENT**6.9. Cyclic AMP-Release Bioassay**

The cAMP assay is a competitive immunoassay commercially available through Amersham Corporation.

Materials and Methods. To determine cAMP release induced by RLF, normal human endometrial cells are grown at 1.2×10^4 cells/well in a 96-well plate in DMEM/F12 + 10% newborn calf serum. 24 hours later, the cells are washed in serum-free medium comprised of DMEM/F12+ 0.2% lactalbumin hydrolysate. 24 hours later, the cells are treated with relaxin and/or RLF in the presence of isobutylmethylxanthine and forskolin for 30 min. The cell lysates are harvested with 0.1N HCL, neutralized with 0.1N NaOH, then assayed in the immunoassay (Amersham Corp).

Experimental Results. When relaxin was assayed at 0.78ng/ml, 86 pM cAMP was measured in endometrial cell lysates. When RLF (2.5 ug/ml) was simultaneously added, 470pM cAMP, roughly a 5-fold enhancement in cAMP production, was measured. When a relaxin concentration of 3.12ng/ml was tested with and without RLF (2.5ug/ml), a 2-fold enhancement was measurable with relaxin + RLF as compared with relaxin alone.

6.10. Mouse Symphysis Pubis Assay

Mouse interpubic ligament assays were performed essentially as described by Steinetz, et al., 1960, *Endocrinology* 67:102-115. Ovariectomized virgin female mice were printed with 5 μ g estrogen cypionate in 100 μ l sesame oil. Five days later the mice were injected subcutaneously with human relaxin, RLF, or mixtures of human relaxin and RLF in 100 μ l of 0.1% benzopurpurin 4B. Specifically, groups of five animals received either human relaxin at a suboptimal dose or a mixture of 0.2 μ g, 0.4 μ g and 0.8 μ g human relaxin and 5 μ g of RLF as given in FIG. 8. For negative control 100 μ l of 0.1% benzopurpurin 4B in

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